PATENT
Attorney Docket 061567-5001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Andrew Howard *et al.***

Application No. **09/914,106**

Filed: **August 23, 2001**

Group Art Unit: **1646**

Examiner: **Michael D. Pak**

For: **G Protein-Coupled Receptor Resembling Galanin Receptors**

DECLARATION UNDER 37 C.F.R. 1.132

I, Brian O'Dowd, do hereby make the following declaration:

1. I am an inventor in the above-referenced application.

2. I have reviewed the Office Action dated October 18, 2005 in the above-referenced application, in particular the Examiner's comments concerning the rejection under 35 U.S.C. 101 for the purported lack of either a specific and substantial asserted utility or a well established utility for the invention set forth in claims 14 to 34.

3. The nucleic acid molecule in claims 14 to 34 (SEQ ID NO: 1) encodes the GPR54 gene which is a G-protein-coupled receptor and this receptor is expressed in brain (see Figures 6 and 7). In the specification of the above referenced application, it is stated that agonists and antagonists of the GPR54 receptor are useful in the treatment of sexual disorders (see page 24, line 25). The specification also discloses that gene therapy may be used to introduce DNA encoding GPR54 into the cells of target organs and that this procedure would be useful for the treatment of diseases where it is beneficial to elevate GPR54 activity (see page 28, line 9 to 20).

4. Applicants submit herewith a peer-reviewed journal publication (Seminara *et al.* (2003) attached as Exhibit A) published after the filing date of the present application which supports the role of the GPR54 gene in sexual disorders. This publication demonstrates that the GPR54 receptor is a regulator of puberty, and that mutations in GPR54 gene caused the sexual disorder hypogonadotropic

hypogonadism in humans (see Figure 1 and "Conclusions" on page 1614). Seminara *et al.* disclose that transgenic mice lacking the GPR54 receptor were produced which failed to undergo puberty and mutant males were sterile with very small genitalia (see Figure 5). Mutant female mice also failed to undergo sexual maturation and failed to conceive when paired with fertile males. These females do not progress through the oestrus cycle (see page 1622, first column, third paragraph). These animal studies indicate that the GPR54 receptor is required for a subject to undergo puberty.

5. Applicants also submit herewith a second a peer-reviewed journal publication (Semple *et al.* (2005) attached as Exhibit B) which also identifies mutations in the GPR54 gene as the cause of hypogonadotropic hypogonadism in human subjects.

6. The human clinical data and the murine experimental data disclosed in the attached Exhibits further demonstrate that the GPR54 receptor is required for progression through puberty and that abnormal GPR54 activity results in a sexual disorder. The claimed invention therefore derives a therapeutic utility in that the nucleic acids encoding GPR54 can be used in gene therapy as a means to treat individuals with a sexual disorder (*e.g.*, hypogonadotropic hypogonadism) to increase the expression of this gene in subjects who exhibit reduced or zero expression and activity. Applicants disclose that gene therapy may be used to introduce DNA encoding GPR54 into the cells of target organs and that this procedure would be useful for the treatment of diseases where it is beneficial to elevate GPR54 activity (see page 28, line 9 to 20).

7. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

March 20th, '06
Date

Brian O'Dowd
Brian O'Dowd, Ph.D.

ORIGINAL ARTICLE

The *GPR54* Gene as a Regulator of Puberty

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ABSTRACT

BACKGROUND

Puberty, a complex biologic process involving sexual development, accelerated linear growth, and adrenal maturation, is initiated when gonadotropin-releasing hormone begins to be secreted by the hypothalamus. We conducted studies in humans and mice to identify the genetic factors that determine the onset of puberty.

METHODS

We used complementary genetic approaches in humans and in mice. A consanguineous family with members who lacked pubertal development (idiopathic hypogonadotropic hypogonadism) was examined for mutations in a candidate gene, *GPR54*, which encodes a G protein–coupled receptor. Functional differences between wild-type and mutant GPR54 were examined in vitro. In parallel, a *Gpr54*-deficient mouse model was created and phenotyped. Responsiveness to exogenous gonadotropin-releasing hormone was assessed in both the humans and the mice.

RESULTS

Affected patients in the index pedigree were homozygous for an L148S mutation in *GPR54*, and an unrelated proband with idiopathic hypogonadotropic hypogonadism was determined to have two separate mutations, R331X and X399R. The in vitro transfection of COS-7 cells with mutant constructs demonstrated a significantly decreased accumulation of inositol phosphate. The patient carrying the compound heterozygous mutations (R331X and X399R) had attenuated secretion of endogenous gonadotropin-releasing hormone and a left-shifted dose–response curve for gonadotropin-releasing hormone as compared with six patients who had idiopathic hypogonadotropic hypogonadism without *GPR54* mutations. The *Gpr54*–deficient mice had isolated hypogonadotropic hypogonadism (small testes in male mice and a delay in vaginal opening and an absence of follicular maturation in female mice), but they showed responsiveness to both exogenous gonadotropins and gonadotropin-releasing hormone and had normal levels of gonadotropin-releasing hormone in the hypothalamus.

CONCLUSIONS

Mutations in *GPR54*, a G protein–coupled receptor gene, cause autosomal recessive idiopathic hypogonadotropic hypogonadism in humans and mice, suggesting that this receptor is essential for normal gonadotropin-releasing hormone physiology and for puberty.

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Drs. Seminara, Messager, Chatzidaki, and Thresher contributed equally to the article. Drs. Crowley, Aparicio, and Colledge were the senior authors.

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THE NEUROENDOCRINE AND GENETIC control of sexual maturation at puberty remains one of the great mysteries of human biology. The secretion of gonadotropin-releasing hormone by the hypothalamus represents the first known step in the reproductive cascade — initiating pulsatile release of gonadotropins, gonadal secretion of sex steroids, pubertal development, and gametogenesis. Although the central role of gonadotropin-releasing hormone in the reproductive hierarchy of all mammals is undisputed, little is understood of the genetic factors that modulate its secretion. The identification of such factors is critical for advancing the understanding of normal reproduction and for providing insight into disorders of the pubertal process.

We used complementary genetic approaches in humans and mice to study a gene involved in the onset of puberty. Idiopathic hypogonadotropic hypogonadism in humans is characterized by the absence of spontaneous sexual maturation in the face of concentrations of gonadotropins in the low-normal range. Affected patients have a complete or partial absence of luteinizing hormone pulsations induced by endogenous gonadotropin-releasing hormone[1] and have normal responsiveness to physiological replacement with exogenous gonadotropin-releasing hormone. These observations localize the defect to an abnormality of gonadotropin-releasing hormone synthesis, secretion, or activity.[2-4]

Using linkage analysis and candidate-gene screening, we identified mutations in a G protein–coupled receptor gene, GPR54, in a large, consanguineous Saudi Arabian family with idiopathic hypogonadotropic hypogonadism and in one unrelated black male proband in the United States. Through a complementary approach, we generated *Gpr54*-deficient mice with a phenotype that demonstrated a lack of adult sexual development and low circulating gonadotropin concentrations — features that closely resemble their human counterparts.

METHODS

FAMILY HISTORY

A large Saudi Arabian family in which there had been three marriages between first cousins sought medical attention for infertility (Fig. 1). Six of the 19 offspring (4 men and 2 women) met the standard diagnostic criteria for idiopathic hypogonadotropic hypogonadism (inappropriately low gonadotropin concentrations in the presence of prepubertal concentrations of sex steroids, normal anterior pituitary function, and normal findings on imaging of the brain) and had responsiveness to exogenous, pulsatile gonadotropin-releasing hormone, as previously reported.[5] The collection of blood samples for genetic studies and the clinical protocols were approved by the Subcommittee on Human Studies of the Massachusetts General Hospital, and all participants provided written informed consent.

MUTATION ANALYSIS

Linkage to a 1.06-Mb interval on chromosome 19p13.3 was previously demonstrated.[6] Mutation analysis of candidate genes, beginning with GPR54, was initiated with the use of DNA extracted from whole blood. The sequence of GPR54 complementary DNA (cDNA) (GenBank accession number AY253981) was aligned with the published genomic sequence[7] to identify the genomic structure. Details of polymerase-chain-reaction (PCR) amplifications, sequencing, control populations, transient transfections, the targeting construct, and biochemical assays are provided in Supplementary Appendix 1 (available with the full text of this article at http://www.nejm.org).

ADDITIONAL POPULATIONS

To determine whether the observed base-pair changes in GPR54 were normal variants, control populations of 80 North American persons (primarily anonymous blood donors), 50 Middle Eastern persons, and 50 black persons from North America were also screened. An additional 63 patients with normosmic idiopathic hypogonadotropic hypogonadism and 20 patients with anosmic idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome) were also screened for coding-sequence mutations in GPR54. Six patients with normosmic idiopathic hypogonadotropic hypogonadism who had participated in dose–response studies of exogenous, pulsatile gonadotropin-releasing hormone and who were negative for GPR54 mutations on genomic screening were selected for comparisons of genotypes and phenotypes.

ALLELE-SPECIFIC CLONING

To demonstrate that there were base-pair changes on separate alleles, specific PCR products were cloned into a pCRII-TOPO plasmid vector (Invitrogen). Colonies were grown, and their DNA sequenced.

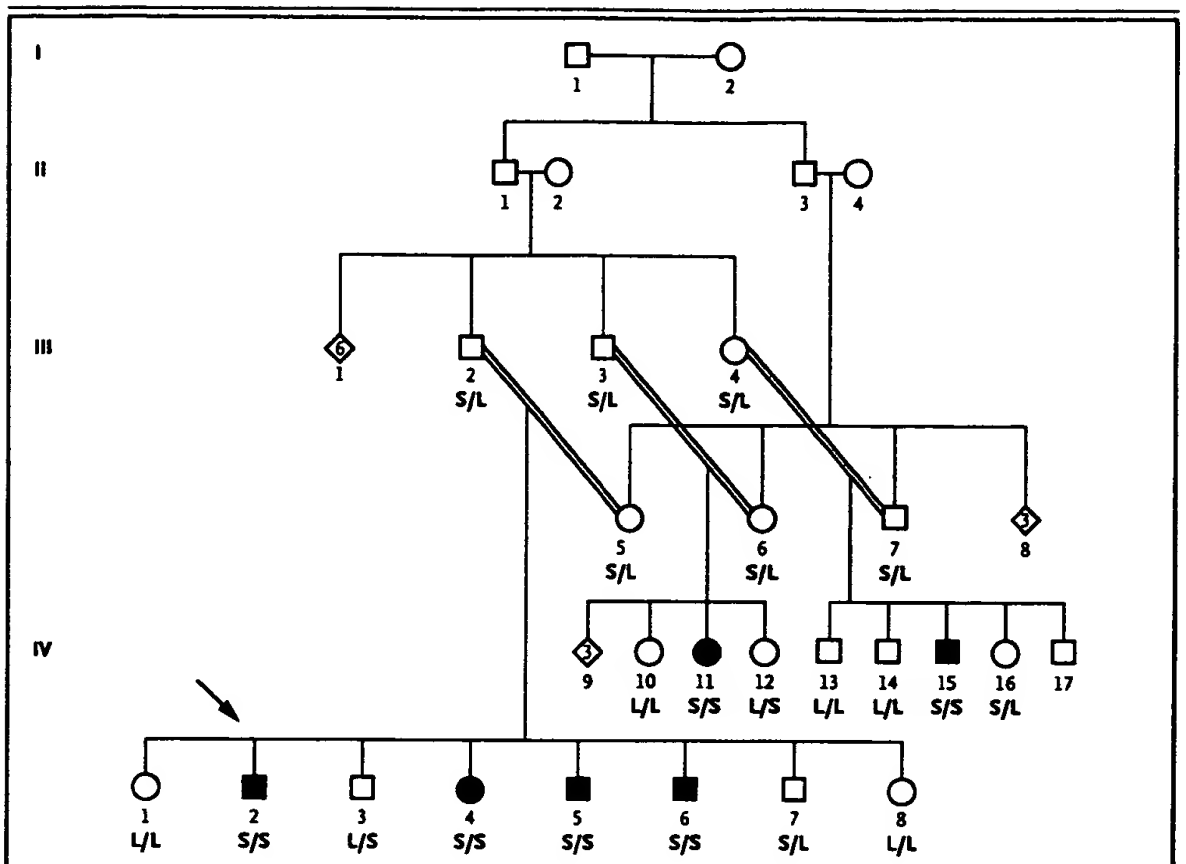

Figure 1. Pedigree of a Family with Idiopathic Hypogonadotropic Hypogonadism.

Squares represent male family members, circles female family members, diamonds multiple family members with the number given within, and solid symbols affected family members; the proband is indicated with an arrow. L denotes leucine, and S serine at position 148 of the *GPR54* gene. Adapted with permission from Bo-Abbas et al.[5] and Acierno et al.[6]

REVERSE-TRANSCRIPTASE PCR

Subjects in whom coding sequence changes were identified in GPR54 were further screened by means of reverse-transcriptase (RT)–PCR to rule out cryptic splicing events. Total RNA was extracted from lymphoblastoid cell lines, and GPR54 cDNA was amplified and sequenced.

GENERATION OF MUTANT CONSTRUCTS

The sequence of the mammalian expression vector pCMVsport 6 containing full-length wild-type GPR54 (clone CS0DE005YC17, Invitrogen) was confirmed by direct sequencing and found to contain a polyA tail. Site-directed mutagenesis was performed to introduce the three mutations (L148S, R331X, and X399R) into this vector. In addition, a stop codon was introduced immediately after the polyA tail (the construct was called "X399R polyA stop").

STUDIES OF GPR54 SIGNALING

A natural ligand for GPR54, kisspeptin-1 (encoded by the gene *KISS1*), has been identified by three separate groups.[8-10] Its C-terminal decapeptide kisspeptin-1 112–121[9] was demonstrated to be the minimal-length peptide required for the full stimulation of GPR54 (the Gq class of G proteins, coupled to phospholipase C). Stimulation of GPR54 by kisspeptin-1 112–121 has been shown to increase phosphatidylinositol turnover.[10]

Kidney (COS-7) cells from African green monkeys were transiently transfected with 1.5 μg of each GPR54 construct or empty vector (pCMVsport6) per well, were stimulated with varying doses of kisspeptin-1 112–121 for 45 minutes, and were subsequently extracted with 20 mM of formic acid. Supernatants were loaded onto anion-exchange columns, and inositol phosphates were extracted.[11] Assays were performed in triplicate.

QUANTITATIVE RT-PCR

Quantitative RT-PCR was performed on RNA isolated from immortalized lymphoblasts obtained from patients (TaqMan One-Step RT-PCR Master Mix, Applied Biosystems). Different primers and probes capable of specifically amplifying the R331X and X399R alleles were used. Samples were run in quadruplicate in a minimum of two independent experiments. The β-actin gene was used as an endogenous control to standardize the assays in terms of expression levels.

GENOTYPE–PHENOTYPE CORRELATIONS

The patient carrying mutations R331X and X399R was admitted to the General Clinical Research Center of the Massachusetts General Hospital. Blood sampling was performed every 10 minutes for 12 hours. The patient then received gonadotropin-releasing hormone subcutaneously every two hours, and his dose was titrated while he was an outpatient until his pituitary–gonadal axis had normalized. After 11 months of treatment, the patient underwent a dose–response study in which four doses of gonadotropin-releasing hormone spanning 1.5 logarithmic orders (7.5 to 250 ng per kilogram of body weight per bolus) were administered intravenously in random order, and luteinizing hormone was sampled frequently.[12] Pulsatile hormone secretion was assessed with the use of the modified version of the method of Santen and Bardin.[13,14]

STUDIES IN MICE

The transgenic mice (*Gpr54*^{tm1PTL}) were maintained as an inbred stock on a 129S6/SvEv genetic background. The gene-targeting strategy engineered a germ-line deletion of transmembrane loops 1 and 2 and the encompassing domains (Fig. 2A). Correct targeting was verified for the 3′ and 5′ arms by Southern blotting and PCR (Fig. 2B). The generation of a null *Gpr54* allele was confirmed by RT-PCR (Fig. 2C). Genotyping was performed by PCR (see Supplementary Appendix 2, available with the full text of this article at http://www.nejm.org). All experiments were performed under the authority of a U.K. Home Office Project License and were approved by a local ethics panel.

Gonadotropin-Releasing Hormone Injection

Wild-type female mice were staged with the use of vaginal smears. Wild-type female mice at diestrus and *Gpr54* –/– female mice received four intraperitoneal injections of 25 ng of gonadotropin-releasing hormone (Sigma) at 30-minute intervals.[15] The mice were killed 30 minutes after the last injection. Blood samples and pituitary specimens were treated as previously described,[15] except that the pituitary specimens were homogenized in 0.3 ml of phosphate-buffered saline.

Hormone Assays

The sensitivity of the immunoradiometric assay for luteinizing hormone was 0.07 ng per milliliter (intraassay variation, 6.0 percent; interassay variation, 12.5 percent), and the sensitivity of the radioimmunoassay for follicle-stimulating hormone was 2 ng per milliliter (intraassay variation, 10 percent; interassay variation, 18 percent). Gonadotropin-releasing hormone was measured by radioimmunoassay,[16] with a detection limit of 0.2 pg per tube (0.83 pg per milliliter) and an intraassay variation of 13 percent. Testosterone was measured by radioimmunoassay, with a sensitivity of 0.2 nmol per liter (intraassay variation, 6.0 percent; interassay variation, 18 percent). 17β-estradiol was measured by enzyme-linked immunosorbent assay (ELISA), with a sensitivity of 10 pg per milliliter (intraassay variation, 3.9 percent; interassay variation, 10 percent).

Histologic Studies

Mouse tissues were dissected and fixed for four hours in 4 percent formaldehyde and were then washed three times in 0.01 percent phosphate-buffered saline. Ovaries, testes, and adrenal glands were wax-embedded and sectioned at 3 to 4 μm. Tissue sections were stained with hematoxylin and eosin. Mammary glands were dissected and fixed for 2 to 4 hours at 23°C in fixative (six parts absolute ethanol to three parts chloroform to one part glacial acetic acid), washed in 70 percent ethanol for 15 minutes, rehydrated, and stained overnight in carmine alum stain that has been boiled for 20 minutes in 500 ml of distilled water. Slides were washed in increasing concentrations of ethanol (70 percent, 95 percent, and 100 percent) for 15 minutes each, cleared in xylene for 30 minutes, and mounted.

RESULTS

MUTATION ANALYSIS

Linkage in a consanguineous Saudi Arabian family was previously demonstrated on chromosome 19p13.3 with a maximal two-point lod score of

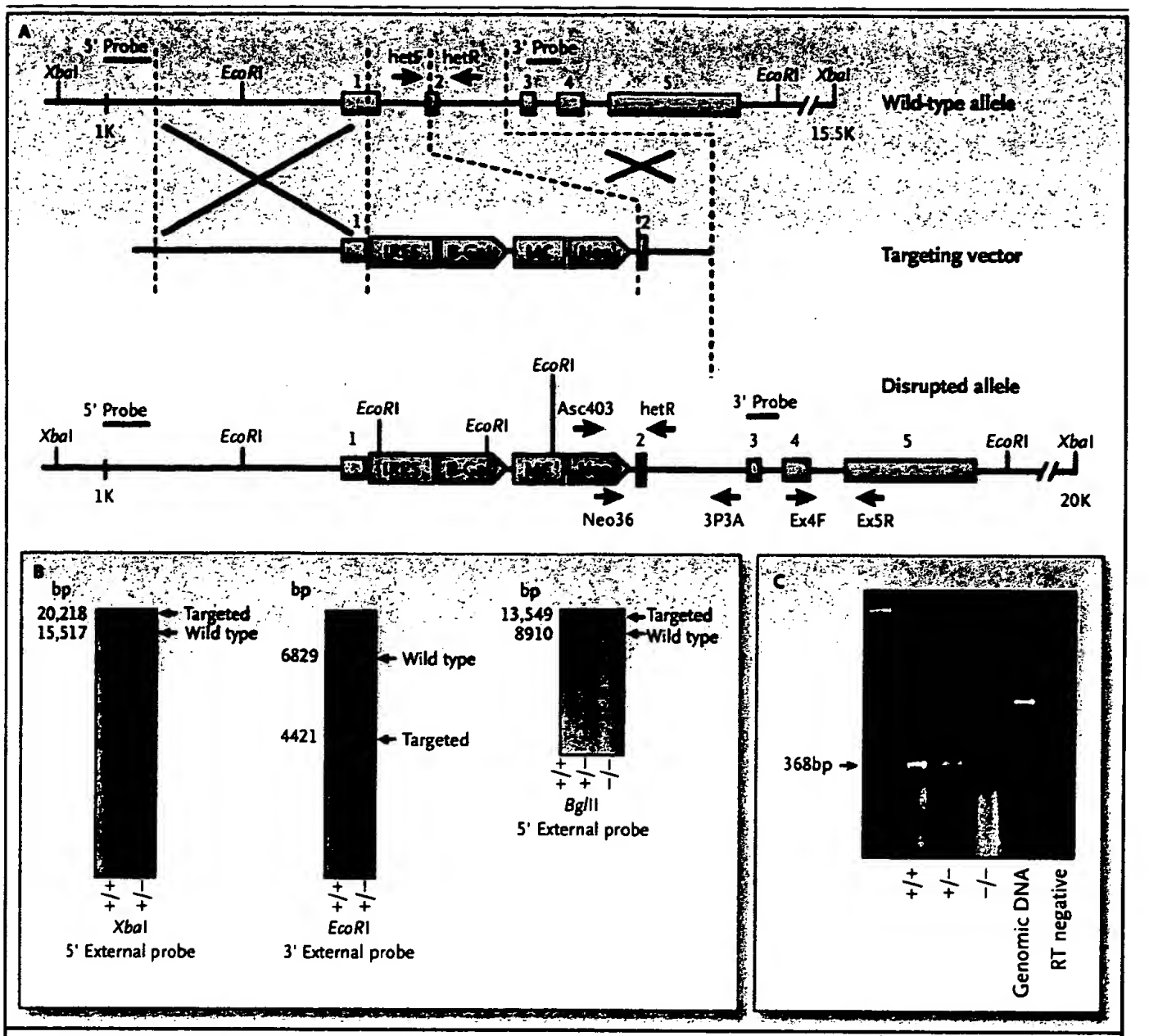

Figure 2. Targeted Deletion in the *Gpr54* Locus in Mice.

Panel A shows a schematic representation of the allele of *Gpr54* that was targeted for deletion (Panel A). Blue boxes represent exons, and purple boxes resistance cassettes and markers. Key restriction sites, primers, and probes are shown above the loci. Panel B shows the correct targeting of the 5' and 3' arms as demonstrated by Southern blotting. The arrows indicate the mutant and wild-type bands detected by diagnostic restriction digestion and probing. The sizes are given in numbers of base pairs. Southern blots show the expected pattern in heterozygous, wild-type, and mutant mice. In mice that are homozygous for the *Gpr54* deletion (Panel C), transcription 3' of the locus has been ablated. RT-PCR analysis of the segment spanning exons 4 and 5 shows that a detectable transcript is absent. IRES denotes internal ribosome entry site, B-Gal beta-galactosidase gene, MC MC promoter, Neo neomycin resistance gene, and Ex exon.

5.17.[6] The candidate region on chromosome 19 contained 23 known genes,[7] including GPR54, which is expressed in the human brain, pituitary gland, and placenta, as assessed with the use of RT-PCR.[9,10] GPR54 has five exons and contains an open reading frame of 1197 bp that encodes a 398-amino-acid protein.

A homozygous single-nucleotide variant (443T>C) in exon 3, which substitutes a serine for the normal leucine at position 148 (L148S) in the second intracellular loop, was found in all six affected persons in the Saudi pedigree and did not occur in a homozygous state in any unaffected family members (all references to base-pair positions are

reported according to standard numbering and nomenclature[17]) (see Supplementary Appendix 3, available with the full text of this article at http://www.nejm.org). This variant does not appear to be a polymorphism, since it occurs only in affected family members; is absent in 160 chromosomes from unrelated, unaffected controls from the United States and 100 chromosomes from controls from the Middle East; is present in an amino acid residue that is conserved among species including mouse, rat, amphioxus, and pufferfish (GenBank accession numbers AF343726, BAB55447, and AAM18884, and Fugu Genome Server[18] accession number SINFRUP00000071513,[19] respectively); and changes the polarity of the encoded amino acid from hydrophobic to neutral.

Of the 63 unrelated patients with normosmic idiopathic hypogonadotropic hypogonadism and the 20 patients with Kallmann's syndrome, one black man with idiopathic hypogonadotropic hypogonadism was discovered to have a heterozygous C-to-T transition at nucleotide 991 in exon 5, in which an arginine at residue 331 was replaced with a premature stop codon (991C>T [R331X]). In addition, a heterozygous T-to-A transversion was identified at nucleotide 1195 in exon 5, which replaced the stop codon at residue 399 with an arginine (1195T>A [X399R]) (Supplementary Appendix 3). This nonstop mutation results in the continuation of the open reading frame to the polyA signal, with no intervening stop codon. Neither change was identified in the 160 chromosomes from North American controls or the 100 chromosomes from black controls.

To confirm that the nonsense and nonstop mutations are found on separate chromosomes, allele-specific cloning was performed. Seventeen clones contained either R331X or X399R, and no clones contained both, confirming that the two variants occur on separate alleles (making the patient a compound heterozygote) (data not shown).

RT-PCR

RT-PCR products generated from the Saudi Arabian proband and the compound heterozygote were of the expected size for all segments. Sequence analysis of these products revealed that the newly identified mutations did not result in cryptic splicing (data not shown).

FUNCTIONAL ASSAYS

To determine whether the identified changes in GPR54 affect the function of the receptor, inositol phosphate production was measured in COS-7 cells in response to kisspeptin-1 112–121. The maximal inositol phosphate response of the cells that were transfected with the mutant L148S and R331X constructs was decreased by 65 percent and 67 percent, respectively, as compared with the cells that were transfected with the wild-type gene (Fig. 3A and 3B). RT-PCR of COS-7 cells transfected with the X399R construct revealed a transcript that contained 3' untranslated region, polyA tail, and expression-vector sequence (data not shown). In the absence of the physiologic stop codon at position 399 (but with a stop codon in the vector sequence), the in vitro transcript resulted in an elongated receptor protein. Because this in vitro construct did not accurately mimic in vivo physiology, it was not used in the functional studies. The X399R polyA stop construct, which makes a protein identical to that encoded by the nonstop transcript, stimulates inositol phosphate production that is 61 percent of that of wild-type GPR54 (Fig. 3C). No inositol phosphate stimulation was observed with pCMVsport 6.

QUANTITATIVE RT-PCR

Expression analysis of the GPR54 alleles was performed by means of real-time PCR, with the use of lymphoblastic messenger RNA (mRNA) as a template. The mutant alleles were expressed at concentrations correlated with concentrations of control lymphoblasts. When compared with the standardized control mRNA, the mean (±SE) total concentration of GPR54 mRNA in the compound-heterozygous patient was 17.6±1.6 percent of the normal concentration ($P<0.001$ by Student's t-test); the expression concentration of the R331X allele was 17.9±1.9 percent of the normal concentration, and the expression concentration of the X399R allele was 2.5±0.3 percent of the normal concentration (Fig. 3D).

ENDOCRINOLOGIC PHENOTYPING

A base-line profile of luteinizing hormone in the proband carrying the heterozygous mutations R331X and X399R is shown in Figure 4A. The patient had low concentrations of luteinizing hormone and low testosterone concentrations, particularly as compared with the mean (±2 SD) luteinizing hormone level measured in 20 normal men.[20,21] Nonetheless, nine low-amplitude pulses of luteinizing hormone are present, as determined by formal pulse analysis. The responses to four doses of intravenous gonadotropin-releasing hormone are compared with the mean amplitude of luteinizing hormone

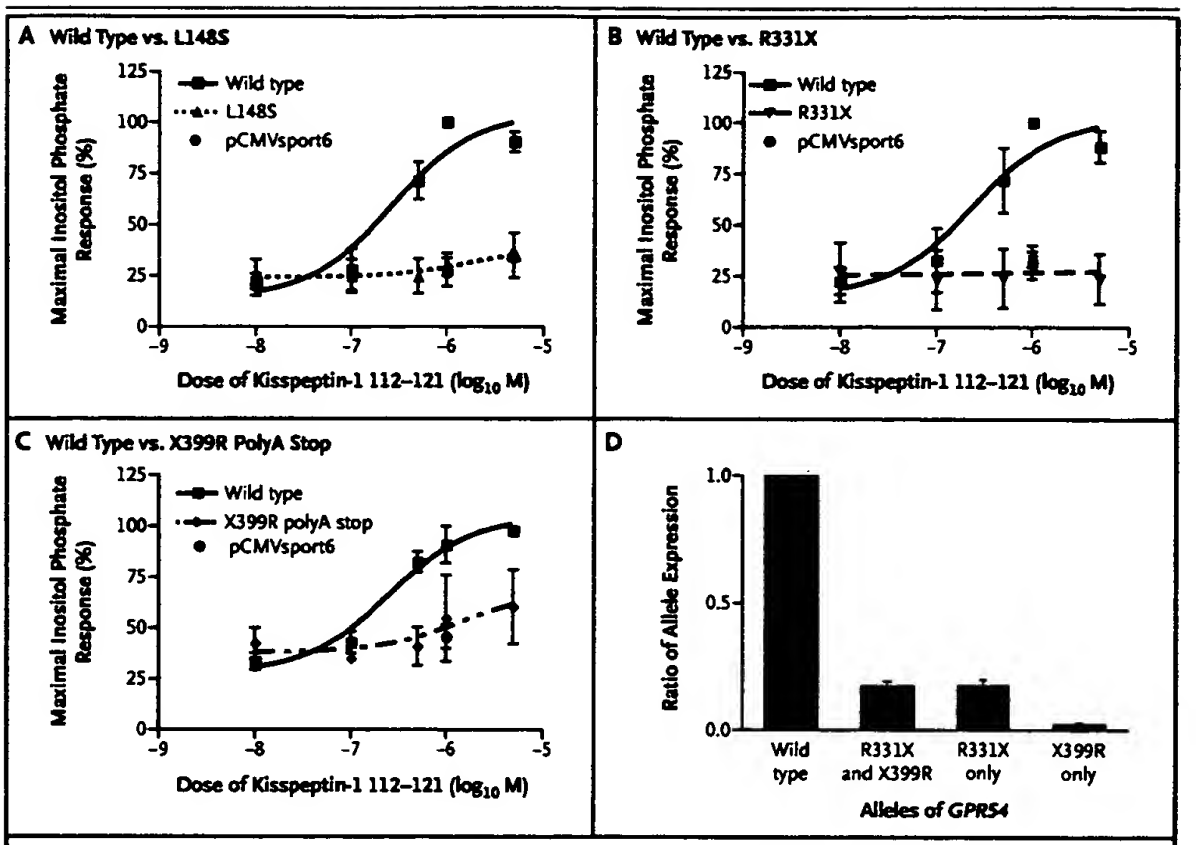

Figure 3. Dose–Response Curves for the Ligand-Stimulated Production of Inositol Phosphate in Mutant Constructs, Corrected for Protein Content.

The data points represent the means of multiple replicates, each measured in triplicate. Cotransfection with beta-galactosidase revealed no differences in the efficiency of transfection; quantitative RT-PCR and Western blotting revealed equivalent transcript and protein expression (data not shown). Panel A shows the curve for the L148S mutation (three independent experiments, each performed in triplicate), Panel B the curve for the R331X mutation (two independent experiments, each performed in triplicate), and Panel C the curve for the X399R polyA stop mutation (two independent experiments, each performed in triplicate); the percentages on the y axis represent the percentages of the maximal stimulation for each *GPR54* construct. Panel D shows the relative quantification of the wild-type and mutant *GPR54* allele expression in lymphoblastoid cell lines as measured by quantitative RT-PCR. I bars represent standard errors.

pulses in six other patients with idiopathic hypogonadotropic hypogonadism who were treated with the same regimen (Fig. 4B and 4C). The dose–response curve for the proband is shifted leftward as compared with the 95 percent confidence intervals around the mean responses in men with idiopathic hypogonadotropic hypogonadism who did not have any GPR54 mutations.

PATHOPHYSIOLOGY, ANATOMY, AND BEHAVIOR OF HOMOZYGOUS *GPR54*-DEFICIENT MICE

Homozygous mutant mice (*Gpr54* –/–) (Fig. 2A and 2B) were viable and obtained at the expected mendelian frequency from heterozygous breeding pairs (Fig. 2B and Supplementary Appendix 2). RT-PCR analysis of transcripts showed no detectable transcription in the 3′ end of the homozygous *Gpr54*^{tm1PTL} allele (Fig. 2C). *Gpr54* +/– mice were phenotypically normal and were fertile. *Gpr54* –/– mice did not display any of the physiologic changes associated with sexual maturation. The testes of male *Gpr54* –/– mice were significantly smaller than those of age-matched controls (Fig. 5A) (mean weight in nine *Gpr54* –/– mice, 0.05±0.00 g; mean weight in eight age-matched controls, 0.18±0.01 g; $P<0.001$ by the unpaired Mann–Whitney U test) and did not contain spermatozoa in the lumen of the seminiferous tubules or the epididymis (Fig. 5C, 5D, 5E, and 5F). Primary spermatocytes were present, but there were very few haploid spermatids, which suggests that spermatogenesis had been initiated but had stopped before the meiotic-division stage.

Male mice also lacked development of second-

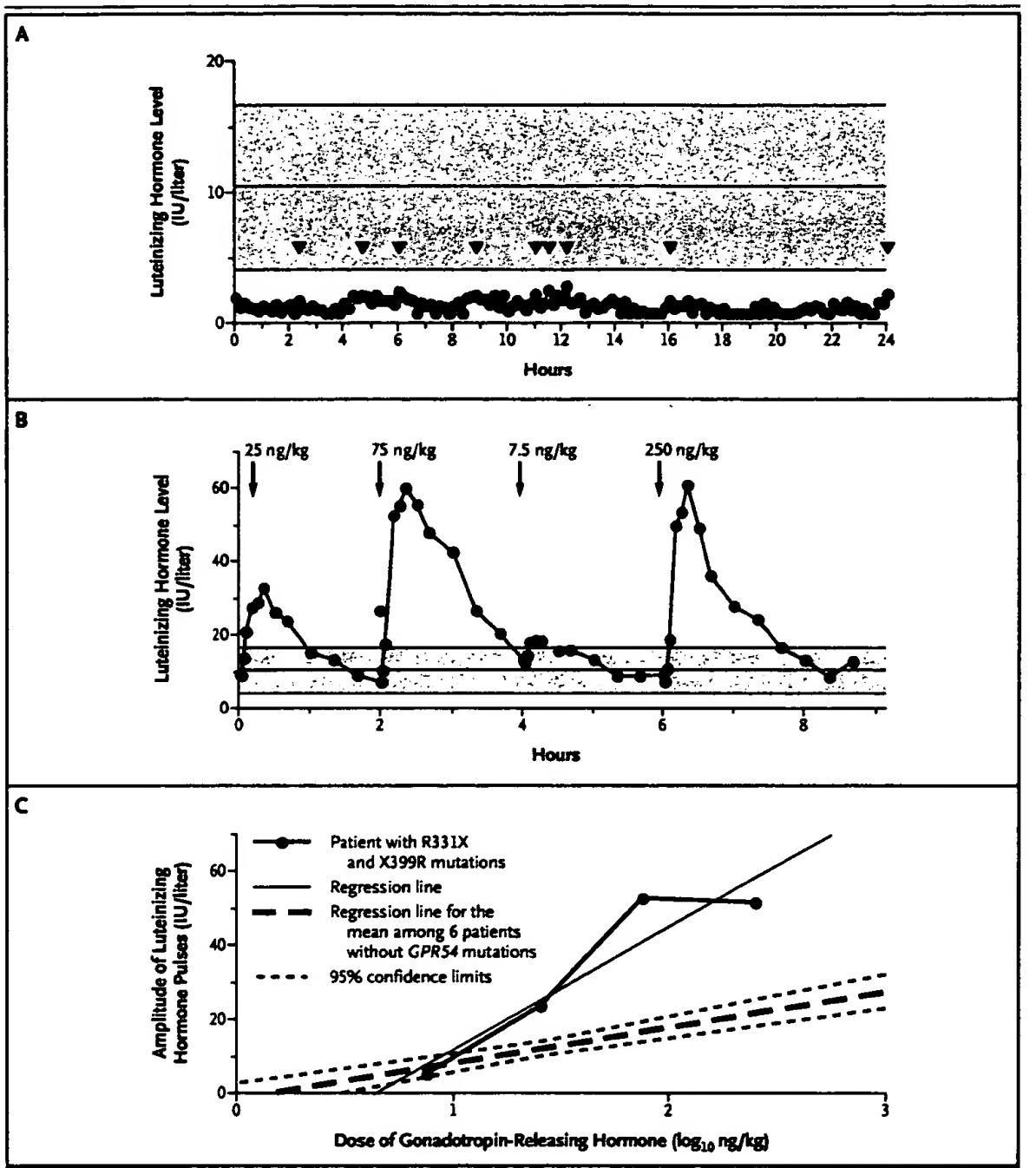

Figure 4. Biochemical Phenotyping in a Patient Carrying the *GPR54* Mutations R331X and X399R.

Panel A shows the base-line pattern of luteinizing hormone secretion, with measurements of the gonadotropin at 10-minute intervals over a 24-hour period. The pooled luteinizing hormone concentration was 2.0 IU per liter, the pooled follicle-stimulating hormone concentration 3.9 IU per liter, and the pooled testosterone concentration 1.22 nmol per liter (normal range, 11.42 to 33.6); the testicular volume was 1 ml for each testis. The shaded areas in Panels A and B represent the mean concentration (±2 SD) of secretion of luteinizing hormone among 20 normal men. Arrowheads represent pulses. Panel B shows the luteinizing hormone response to exogenous pulsatile gonadotropin-releasing hormone administered intravenously in a range of doses. The doses were administered in random order. The pooled testosterone concentrations ranged from 20.90 to 23.65 nmol per liter; the testicular volume was 6 ml for the right testis and 8 ml for the left testis. The patient received exogenous pulsatile gonadotropin-releasing hormone subcutaneously every two hours on an outpatient basis for 11 months before the study began. Panel C shows the dose–response curve for the patient, drawn with a regression line. The data for the patient fall to the left of the 95 percent confidence intervals around the mean amplitudes of the luteinizing hormone response in six other men with idiopathic hypogonadotropic hypogonadism who were receiving the same regimen.

ary sex glands, including the preputial gland (Fig. 5G and 5H), the seminal vesicles, and the prostate (not shown). In the adrenal glands of the mutant animals, the innermost region of the cortex, which normally regresses at puberty, was still present (Fig. 5I and 5J). Sexual mounting behavior was also not observed among the male mice. No gross morphologic abnormalities were found in the central nervous system of *Gpr54* –/– mice, and the mutant mice thrived, apart from the reproductive defect.

Female mutant mice also had defective sexual development; they had small vaginal openings and did not become pregnant after appropriate mating exposure. Vaginal smears consisted of nonkeratinized epithelia and mucus strands similar to those observed in immature female mice, indicating the lack of an estrus cycle. The uterine horns in female *Gpr54* –/– mice were threadlike, and the ovaries were significantly smaller than normal (mean weight in nine wild-type mice, 5.7±0.7 mg; mean weight in eight mutant mice, 1.0±0.1 mg; $P<0.001$ by the unpaired Mann–Whitney U test) (Fig. 5B). Mammary tissue showed no postpubertal maturation of branched epithelial ducts (Fig. 5K and 5L). The ovaries contained primary and secondary follicles and occasionally an early antral follicle but no large graafian follicles or corpora lutea (Fig. 5M and 5N).

ENDOCRINOLOGIC PHENOTYPES IN *GPR54*-DEFICIENT MICE

Male *Gpr54* –/– mice had significantly lower blood testosterone concentrations than age-matched +/+ controls (mean among 12 mutant mice, 0.1±0.02 pg per milliliter; mean among 11 wild-type mice, 4.6±1.6 pg per milliliter; $P<0.001$ by the unpaired Mann–Whitney U test). The testosterone concentrations in male *Gpr54* –/– mice were similar to those observed in female *Gpr54* +/+ mice (mean among eight female mice, 0.2±0.02 pg per milliliter) (Fig. 6A). The 17β-estradiol concentrations in female *Gpr54* –/– mice were similar to those in *Gpr54* +/+ females at nonestrus stages of the reproductive cycle (Fig. 6B) and to the base-line serum estradiol concentrations in male *Gpr54* +/+ mice (data not shown). No *Gpr54* –/– females were identified that had a 17β-estradiol concentration similar to that found at estrus (mean concentration among five wild-type females, 96.5±16.3 pg per milliliter) (Fig. 6B).

The lack of an estrus cycle in female mice was not caused by an inability of gonadal tissue to respond to gonadotropins. Female *Gpr54* –/– mice could be induced to ovulate after sequential injection of the gonadotropins pregnant mares serum and human chorionic gonadotropin (data not shown). The lack of an estrus cycle and the failure to produce sperm in *Gpr54* –/– mice were caused by a significant reduction in the serum follicle-stimulating hormone concentration ($P=0.009$) and a more moderate decrease in the luteinizing hormone concentration (Fig. 6C and 6D). Possible explanations of the reduced concentrations of circulating gonadotropins include an absence of pituitary gonadotropes, an inability of existing gonadotropes to respond to stimulation by gonadotropin-releasing hormone, and a lack of gonadotropin-releasing hormone production. This last possibility was ruled out because there was no significant difference between normal and mutant mice in the concentration of gonadotropin-releasing hormone in hypothalamic extracts (Fig. 6E).

In addition, measurements of pituitary luteinizing hormone and follicle-stimulating hormone showed that although the total amount of each hormone was lower in *Gpr54* –/– mice than in wild-type mice, significant quantities of each hormone were found, indicating that the pituitary gonadotropes are present in *Gpr54* –/– mice and are capable of synthesizing luteinizing hormone and follicle-stimulating hormone. Furthermore, in adult female *Gpr54* –/– mice, luteinizing hormone was secreted into the bloodstream in response to the injection of gonadotropin-releasing hormone (Fig. 6F), and there was a corresponding depletion in pituitary luteinizing hormone (Fig. 6G). Studies of the secretion of follicle-stimulating hormone in response to the injection of gonadotropin-releasing hormone had similar results (data not shown). Although the absolute concentration of serum luteinizing hormone after the injection of gonadotropin-releasing hormone was lower in *Gpr54* –/– mice than in +/+ mice, the proportional increase in the luteinizing hormone concentration was similar (an increase by a factor of five from base line). These responses are consistent with a first exposure to gonadotropin-releasing hormone.[22,23]

DISCUSSION

In primates, the hypothalamic–pituitary–gonadal axis is fully active during neonatal life, followed by a mysterious period of dormancy during childhood. The triggers of the onset of gonadotropin-releasing hormone secretion at puberty are as unclear as

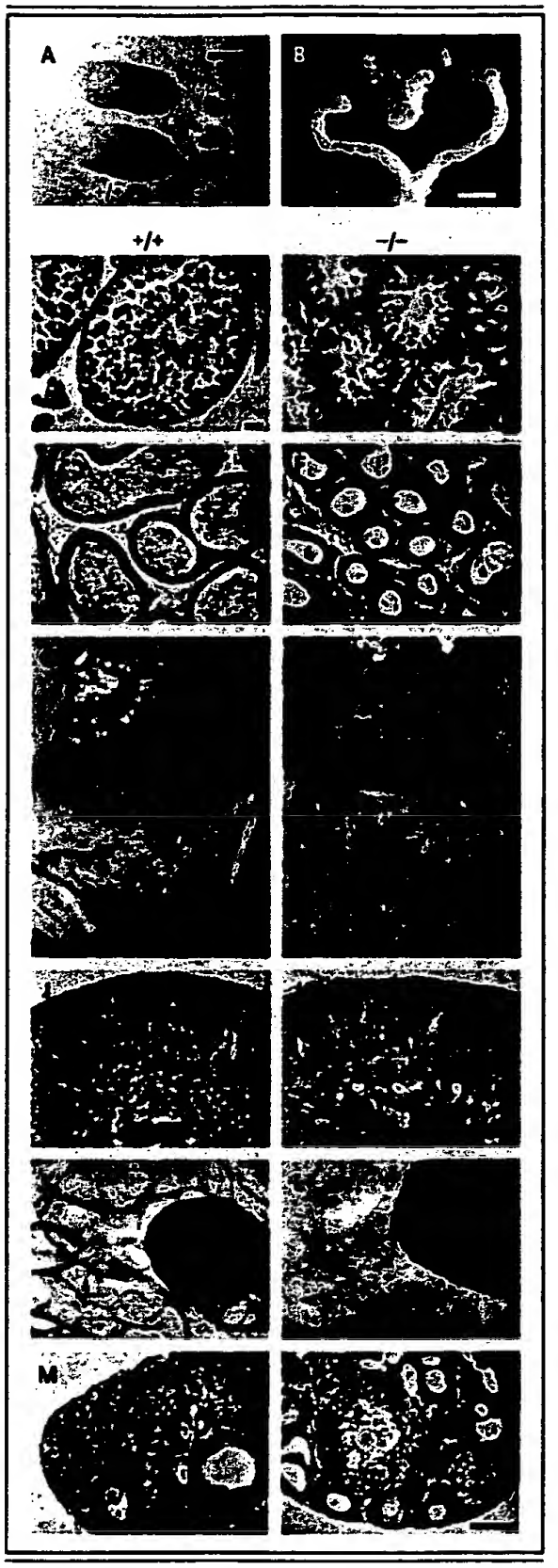

Figure 5. Gonadal Anatomy and Secondary Sexual Characteristics of *Gpr54* −/− Mice.

Panel A shows the reduction in the size of the testes (wild-type as compared with mutant male mice), and Panel B shows the small ovaries and uteri found in female *Gpr54* −/− mice; the scale bars represent 0.5 cm. In Panels C through N, the wild-type mouse is represented by the left-hand column and the mutant mouse is represented by the right-hand column. Panel D shows the reduction in the number of spermatozoa in the seminiferous tubules, as compared with Panel C; the scale bars represent 50 μm. Panels E and F show the presence and absence, respectively, of sperm in the epididymis; the scale bars represent 100 μm. Panel H shows reduced development of the preputial gland, as compared with Panel G; the scale bars represent 1 cm. Panels I and J show the absence and presence, respectively, of the prepubescent zone X in the adrenal gland; the scale bars represent 20 μm. Panel L shows reduced mammary-duct formation, as compared with Panel K (the dark mass is lymph node); the scale bars represent 0.5 cm. Panels M and N show the presence and absence, respectively, of graafian follicles and corpora lutea; CL denotes corpus luteum; the scale bars represent 300 μm.

those that halt its secretion at the end of the neonatal period. Insight into this process has been gained through the study of various diseases in humans and animal models in which genetic defects cause abnormalities of sexual maturation. Mutations in both GPR54 in humans and Gpr54 in mice cause hypogonadotropic hypogonadism, pubertal delay, and sexual infantilism that can be corrected by the administration of exogenous gonadotropin-releasing hormone. Taken together, these observations establish that the effect of GPR54 on gonadotropin-releasing hormone secretion is conserved in multiple mammalian species and is a genetic determinant of sexual maturation.

GPR54 is a member of the rhodopsin family of G protein–coupled receptors whose sequences are most similar to those of members of the galanin-receptor family (35 to 40 percent identity).[9] Although galanin and galanin-like peptide appear not to bind to GPR54,[9,24] endogenous peptides derived from a precursor protein, kisspeptin-1, have recently been identified that do display agonist activity.[8-10] The longest of these peptides is kisspeptin-1 68–121, or metastin, so called because of its ability to suppress metastatic potential in melanoma and breast-cancer cell lines.[25-27] Metastin is secreted into the circulation by the placenta in relatively large quantities throughout gestation, although its physiologic role in pregnancy remains unknown.[28]

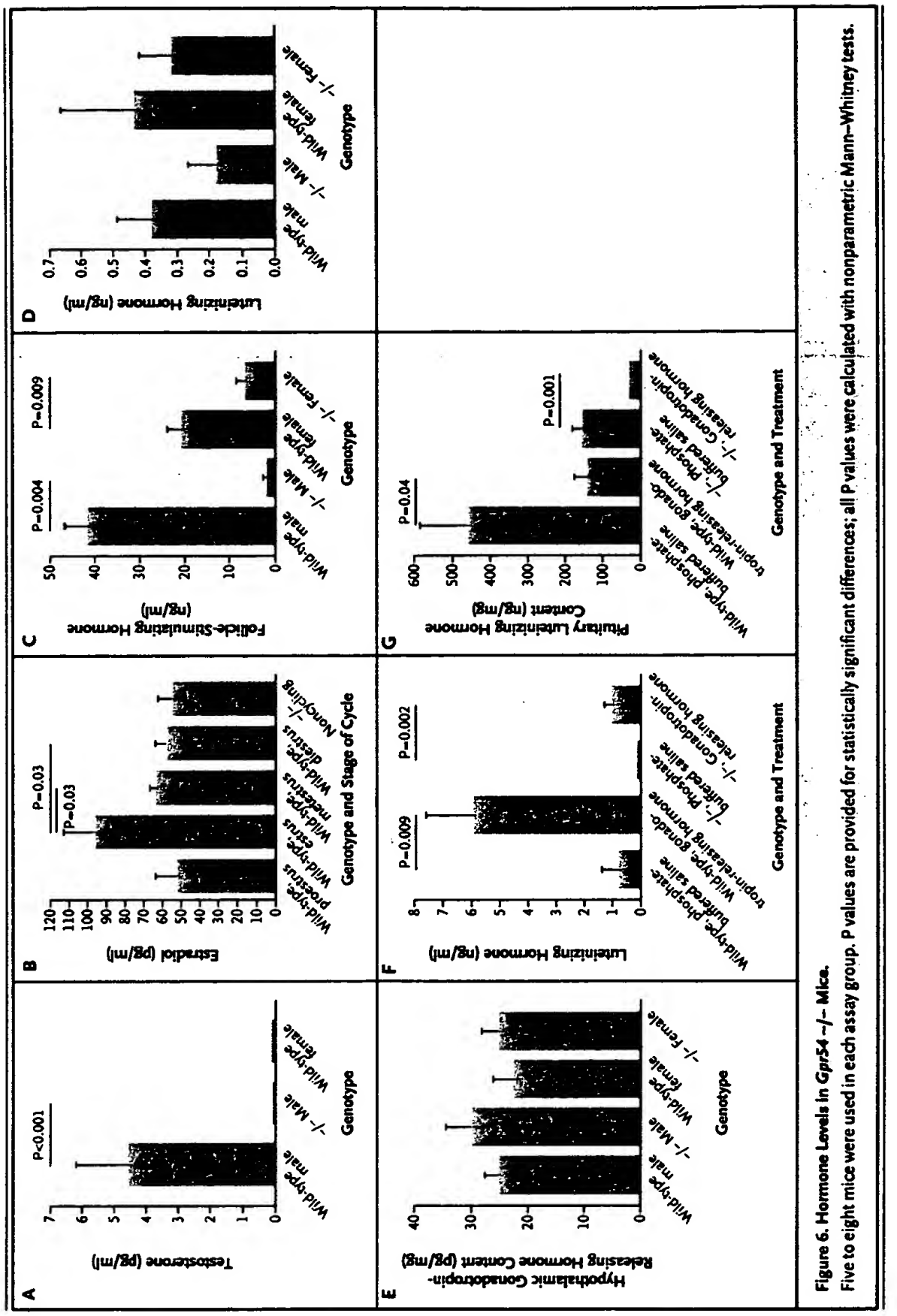

Figure 6. Hormone Levels in *Gpr54* -/- Mice.

Five to eight mice were used in each assay group. P values are provided for statistically significant differences; all P values were calculated with nonparametric Mann–Whitney tests.

In this study, a variety of mutations in GPR54 were identified in patients with idiopathic hypogonadotropic hypogonadism. The index family was found to carry a homozygous L148S substitution. When expressed in cell lines, the L148S mutant construct markedly reduced inositol phosphate production as compared with the wild-type construct. A male patient with sporadic idiopathic hypogonadotropic hypogonadism was also found to be a compound heterozygote for the mutations R331X and X399R, which gave rise to nonsense and nonstop transcripts. It has been shown that mRNAs with premature termination codons can be targeted to nonsense-mediated decay,[29] whereas mRNAs without an in-frame termination codon can be subject to nonstop decay, a recently identified degradation pathway that is initiated when the ribosome reaches the 3′ terminal of the mRNA.[30,31]

We hypothesized that this combination of nonstop and nonsense mutations in GPR54 in the patient with sporadic idiopathic hypogonadotropic hypogonadism would result in the absence of a functional receptor. Quantitative RT-PCR confirmed the presence of dramatically reduced concentrations of GPR54 mRNA in the patient with the R331X and X399R alleles. Moreover, this total concentration appeared to be composed almost exclusively of the nonsense transcript. These results indicate that the contribution of the nonstop transcript was almost negligible, supporting the hypothesis of nonstop decay. In the unlikely event that a protein were produced by the X399R transcript, our findings regarding the X399R polyA stop construct suggest that it would function poorly.

The clinical phenotype of the patient carrying the R331X and X399R mutations was associated with a neuroendocrine profile involving low-amplitude pulses of luteinizing hormone, suggesting reduced secretion of gonadotropin-releasing hormone. This notion is supported by the leftward-shifted dose–response curve as compared with those for other patients with idiopathic hypogonadotropic hypogonadism who were undergoing the same therapy, suggesting that this patient was more sensitive to exogenous gonadotropin-releasing hormone. Since these studies were performed, deletions in GPR54 have been described in a separate family with idiopathic hypogonadotropic hypogonadism, although the phenotypic features of this family were not detailed.[32] Although the investigators in that case agree with our conclusion that the frequency of *GPR54* mutations as a cause of idiopathic hypogonadotropic hypogonadism is low (4 of 113 total cases), GPR54 reveals a new direction for the exploration of other genes that are essential for the secretion of gonadotropin-releasing hormone.

The *Gpr54*-deficient mice had striking physiological similarities to the patients with idiopathic hypogonadotropic hypogonadism, including a lack of sexual maturation associated with low concentrations of gonadotropins. In addition, their gonads remained sensitive to exogenous gonadotropins, and their pituitary gonadotropes remained responsive to stimulation by gonadotropin-releasing hormone. This strong similarity between the findings in the patients and those in the mouse model establishes a central role for GPR54 in gonadotropin-releasing hormone secretion and the onset of sexual maturation among mammalian species. Moreover, the use of *Gpr54*-deficient mice permitted the quantitation of their hypothalamic gonadotropin-releasing hormone concentrations, which were normal in the face of their hypogonadotropism. The presence of normal concentrations of gonadotropin-releasing hormone in the hypothalamus of *Gpr54*-deficient, sexually immature mice is reminiscent of prepubertal rats and monkeys who have normal numbers of gonadotropin-releasing hormone–containing neurons, normal mRNA concentrations, and normal concentrations of gonadotropin-releasing hormone in the hypothalamus.[33,34] Extrapolation from the *Gpr54*-deficient mice to nonhuman primates and humans suggests that GPR54 may have a substantial effect on the processing or secretion of gonadotropin-releasing hormone.

There are three possible mechanisms that may allow abnormalities in GPR54 to cause pubertal delay. The first possibility is that defects in the metastin–GPR54 system perturb gonadotropin-releasing hormone neuronal migration that is analogous to the abnormal axonal targeting that occurs in the X-linked form of Kallmann's syndrome (idiopathic hypogonadotropic hypogonadism with anosmia).[35-37] In vitro, the metastin–GPR54 system induces an "adhesive phenotype" with inhibition of chemotaxis, focal adhesions and stress fibers, and phosphorylation of focal adhesion kinase and paxillin.[8] However, the normal content of gonadotropin-releasing hormone in the hypothalamus of *Gpr54*-deficient mice argues that there has been an appropriate migration of the neurons containing gonadotropin-releasing hormone from their origin in the olfactory placode to their destination in the hypothalamus. The possibility remains, how-

ever, that there is a subtle defect in the terminal migration or differentiation of these neurons within the hypothalamus.

The second possibility is that GPR54 modulates the activity of gonadotropin-releasing hormone at the level of the pituitary. The presence of small but detectable pulses of luteinizing hormone induced by gonadotropin-releasing hormone in the patient with the R331X and X399R mutations and his leftward-shifted dose–response curve suggest that pituitary responsiveness in the GPR54-deficient patient is, if anything, enhanced, suggesting that loss-of-function mutations in GPR54 do not diminish the sensitivity of gonadotropes to gonadotropin-releasing hormone.

The third possibility is that GPR54 regulates the release of gonadotropin-releasing hormone at the level of the hypothalamus. This hypothesis is supported by three observations: the low-amplitude pulses of luteinizing hormone in the patient carrying the R331X and X399R mutations, his leftward-shifted dose–response curve, and the normal content of gonadotropin-releasing hormone in the hypothalamus of *Gpr54*-deficient mice. Further studies will be required to determine the precise mechanisms of action within the hypothalamus as well as the physiological functions of the peptide ligands for GPR54.

Currently, it appears that the frequency of GPR54 mutations as a cause of idiopathic hypogonadotropic hypogonadism is not high. However, the elucidation of the role of GPR54 as a regulator of gonadotropin-releasing hormone–related physiology and puberty may well provide a seminal clue, offering a new perspective on other candidate genes that may control sexual maturation and puberty. These include the genes affecting the biosynthesis, processing, and secretion of the putative ligands, kisspeptin–metastin; the transcriptional regulation of GPR54 itself; and the signaling pathways downstream of the gene. Our data from patients with idiopathic hypogonadotropic hypogonadism and a mouse model provide strong evidence that GPR54 is a key regulator of the biology of puberty.

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CLINICAL CASE SEMINAR

Two Novel Missense Mutations in G Protein-Coupled Receptor 54 in a Patient with Hypogonadotropic Hypogonadism

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It has recently been shown that loss-of-function mutations of the G protein-coupled receptor (GPR)54 lead to isolated hypogonadotropic hypogonadism (IHH) in mice and humans. Such mutations are thought to be rare, even within the clinical IHH population, and only a handful of alleles have been described, making further screening of IHH populations imperative. We examined the genes encoding GPR54 and its putative endogenous ligand, kisspeptin-1, for mutations in a cohort of 30 patients with normosmic HH or delayed puberty. One subject with HH, of mixed Turkish-Cypriot and Afro-Caribbean ancestry, was found to be a compound heterozygote for two previously undescribed missense mutations in GPR54: cysteine 223 to arginine (C223R) in the fifth transmembrane helix and arginine 297 to leucine (R297L) in the third extracellular loop. Assessed *in vitro* using a previously described sensitive signaling assay in cells stably expressing GPR54, the C223R variant was found to exhibit profoundly impaired signaling, whereas the R297L variant showed a mild reduction in ligand-stimulated activity across the ligand dose range. These novel mutations provide further evidence that human HH may be caused by loss-of-function mutations in GPR54. (*J Clin Endocrinol Metab* 90: 1849–1855, 2005)

HUMAN GONADAL FUNCTION is under the control of the anterior pituitary hormones LH and FSH, the release of which is, in turn, regulated by pulsatile secretion of hypothalamic GnRH. In the first few weeks after birth, this axis is functional, but by around 6 months of age, it becomes quiescent, thus entering the juvenile pause, which persists until puberty. The first detectable event at the initiation of puberty is an upsurge in the amplitude of GnRH pulses, leading to overnight LH pulsatility and subsequent maturation of the rest of the axis at puberty. The mechanisms of the childhood inhibition of GnRH release, and its eventual reactivation, are poorly understood but appear to include peripheral feedback suppression by sex steroids and the actions of ill-defined intrinsic central nervous system pathways (1).

Genetic defects in the hypothalamo-pituitary-gonadal axis are manifested clinically as hypogonadotropic hypogonadism (HH), with either primary failure to undergo puberty or severe delay in the process. Defects may broadly be categorized into those affecting embryonic migration of GnRH-secreting neurones, which lead to anosmia as well as HH (*e.g.* Kallmann syndrome due to *KAL*, or *FGFR1* mutations), and those affecting the pulsatile release or function of GnRH from neurones that have migrated to their normal position. Patients suffering from the latter are normosmic. Around 40% of patients with autosomal recessive normosmic familial HH in one series were found to have mutations in the GnRH receptor gene, whereas only around 10–17% of sporadic cases are thought to harbor such mutations in *GNRHR* (2, 3).

In the last year, the G protein-coupled receptor (GPR)54 has been shown to be a gatekeeper gene for activation of the GnRH axis based on loss-of-function mutations in mice and humans. In humans, loss-of-function mutations in GPR54 have been described in two consanguineous pedigrees and two isolated subjects with HH (4, 5) (OMIM 604161). In tandem, mice bearing homozygous targeted disruptions of the *Gpr54* gene have been found to exhibit a similar failure of sexual maturation (5). This shows that GPR54 is required for the normal function of this axis and suggests that the ligand, kisspeptin-1, may act as a novel neurohormonal regulator of the GnRH axis. The latter has recently received support from studies showing that peripheral and central administration of kisspeptin provokes GnRH release (6–8).

We now describe the genetic screening of the *GPR54* gene as well as the *KISS1* gene encoding its putative endogenous

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Abbreviations: CDP, Constitutional delay of puberty; GPR, G protein-coupled receptor; hCG, human chorionic gonadotropin; HH, hypogonadotropic hypogonadism; SNP, single nucleotide polymorphism.

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ligand, kisspeptin-1, in a cohort of 30 patients with normosmic HH, and report the clinical phenotype of a nonconsanguineous subject who shows compound heterozygosity for two novel missense mutations in the *GPR54* gene. We have also documented the prevalence of single nucleotide polymorphisms (SNPs) in the *GPR54* and *KISS1* genes, one previously undescribed, in both the study population and a Caucasian control population.

Subjects and Methods

Subjects

Subjects for genetic screening were recruited from the clinics at Great Ormond Street Hospital (London, UK) and Addenbrooke's Hospital (Cambridge, UK). Informed consent was obtained according to procedures approved by the local research ethics committee of each institution. Patient characteristics are outlined in Table 1. Isolated HH was defined as inappropriately low gonadotropin levels together with prepubertal concentrations of sex steroids. It was deemed idiopathic in which no family history of the condition was apparent, and congenital in which undescended testes with or without small penis were noted at birth. Constitutional delay of puberty (CDP) was defined as the absence of signs of puberty at 14 yr of age in boys, this being described as severe if signs were still not apparent at 15 yr. All patients were reportedly normosmic, had otherwise normal anterior pituitary function and no midline facial defects, and showed no evidence of structural brain pathology, where assessed. In all but three cases, the coding sequence of the *GnRHR* gene had been sequenced and found to be normal.

The Caucasian control population used was derived from the Medical Research Council Ely Study cohort, recruited from a population sampling frame with a high response rate (74%), making it representative of an ethnically homogeneous Caucasian population in this area of Eastern England (9, 10). In addition, 50 Afro-Caribbean control subjects were also selected from an independently assembled cohort used in a study of the genetics of obesity. This group was entirely unselected with respect to reproductive and gonadal function, as was a group of 45 Turkish control subjects recruited as part of studies into obesity and renal tubular disease.

Genetic screening

Genomic DNA was isolated from whole blood using a QIAamp blood kit (Qiagen, Crawley, UK), and amplified using a GenomiPhi DNA amplification kit (Amersham Biosciences, Chalfont, UK). PCR was performed using BioTaq (Bioline, London, UK) and carried out as recommended by the manufacturer, with addition of dimethylsulfoxide where indicated. Thirty-five cycles (60 sec at 95 C, 60 sec at the annealing temperature, and 60 sec at 72 C) were performed using a PTC-225 Peltier thermal cycler (MJ Research, Watertown, MA). PCR products were verified electrophoretically and sequenced using ABI BigDye Terminator (version 3.1) reagents with electrophoresis on an ABI Prism 3100-Avant genetic analyzer (PE Applied Biosystems, Foster City, CA). Subsequent sequence analysis was performed using Sequencher software (Gene Codes, Ann Arbor, MI). Primers, annealing temperatures, and concentrations of $MgCl_2$ and dimethylsulfoxide used for PCR are detailed in Table 2, together with sequencing primers where different.

To distinguish compound heterozygosity for the two mutations described from heterozygosity for a double mutation, the *GPR54* exon 4 forward primer and exon 5 reverse primer (Table 2) were used to amplify exons 4 and 5 and the intervening intron using reaction conditions as for exon 5. The PCR product was then cloned into a pGEM-T Easy Vector (Promega, Southampton, UK), and 16 separate clones were sequenced.

TABLE 1. Description of cohort studied

Diagnosis	Sex	Ethnicity
Idiopathic IHH	11 Males[a]	12 Caucasian
	3 Females	1 Turkish
		1 Indian
		1 Turkish/Jamaican
Familial IHH	1 Male	1 Caucasian
	2 Females	1 Sri Lankan
		1 Sudanese
Familial IHH or CDP	2 Males	2 Caucasian
	0 Females	
Familial CDP	8 Males	8 Caucasian
	0 Females	
Idiopathic severe CDP	3 Males	3 Caucasian
	0 Females	

[a] Includes proband described.

IHH, Isolated HH; Familial IHH or CDP, families where one sibling has HH and another has CDP within the same generation.

Creation of GPR54 mutant alleles and in vitro functional assays

Wild-type human GPR54 was amplified from human brain cDNA (Origene Technologies, Rockville, MD) using the GC-Rich PCR kit (Roche Diagnostics, Lewes, UK). The 5′ primer TAATCAAAGCTTGCGATGCACACCGTGGCTACGTC and 3′ primer ACATTAGGATCCTCACTTATCGTCGTCATCCTTGTAATCGAGAGGGGCGTTGTCCTCC (incorporating a 3′ sequence encoding the FLAG epitope tag) were used. The resulting product was cloned into pCRBlunt (Invitrogen, Renfrew, UK) and sequence verified. Stable cell lines expressing the wild-type GPR54-FLAG or one of the two mutant species described were then created using the Flp-In system (Invitrogen). In brief, the GPR54-FLAG was subcloned into pcDNA5/FRT and the point mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All the resulting constructs were verified by direct sequencing. The Flp-In-293 cell line (Invitrogen) was maintained in DMEM supplemented with 10% Fetal Clone III (Hyclone, Cramlington, UK) and 2 mM L-glutamine. To create stable GPR54-expressing cell lines, Flp-In-293 cells were cotransfected with the pcDNA5/FRT-GPR54-FLAG constructs and the Flp recombinase-expressing plasmid pOG44 using polyfect (Qiagen), with subsequent selection by hygromycin B. Transcription of the inserted alleles was confirmed by RT-PCR (data not shown).

To assess signal transduction by the wild-type and mutant receptors, a sensitive calcium mobilization signaling assay was used as previously described (11–13), with calcium flux in response to kisspeptin-1 112–121 (Phoenix Pharmaceuticals, Belmont, CA) measured on a FlexStation using a FLIPR calcium assay kit (both from Molecular Devices, Sunnyvale, CA) in response to Kisspeptin-1 112–121 (Phoenix Pharmaceuticals). Data were analyzed using SoftMax Pro software (Molecular Devices). Experiments were performed in duplicate on 2 consecutive days, and SDS are indicated.

Results

Genetic screening

Sequencing of the coding regions of the *GPR54* (GenBank accession no. AY253981) and *KISS1* (GenBank accession no. AY117143) genes in all 30 patients revealed the presence of two previously described SNPs in the *GPR54* gene, and three previously described SNPs, one novel SNP, and one previously described insertional polymorphism in the *KISS1* gene. The details of these common variants, and their frequency in both the study population and 180 Caucasian control alleles are shown in Table 3.

One boy with isolated HH was found to harbor two rare sequence variants in the GPR54 gene (Fig. 1): the first, a transversion c.667T>C in exon 4, results in substitution of a cysteine near the cytoplasmic end of the fifth transmembrane α-helix for arginine (C223R), whereas the second, a transversion c.891G>T in exon 5, leads to the substitution of an arginine in the third extracellular loop for leucine (R297L). Cloning a section of the GPR54 gene encompassing exons 4

TABLE 2. Primers and reaction conditions used for sequencing

Gene	Exon	Primers (5′–3′)	$[Mg^{2+}]$/mM	%DMSO	T_m
GPR54	1	F: TTC CTG AGT TCC ACA GGC GCA R[a]: AGG TTT CCA TGT GCC ACA CTC seq: AGG GAA AAG ATT CGA CGT TGG	1.5		66
	2	F[a]: GCC CAG CGC CCG CGC ATC R: GTC CCC AAG TGC GCC CTC TC	1.0	5	70
	3	F: CAG GCT CCC AAC CGC GCA G R[a]: CGT GTC CGC CTT CTC CCG TG	1.0	8	64
	4	F[a]: TGG AAA ATG GGC GCA ATA GCT R: TGA AGG TGG TTA GAC GAA AGG	1.5		66
	5	F[a]: GCC TTT CGT CTA ACC ACC TTC R[a]: GGA GCC GCT CGG ATT CCC AC seq1: TTA AGA CCT GGG CTC ACT GC seq2: AGC ACC AGG AAC AGC TGG ATG	1.5	5	66
KISS1	2	F[a]: TCT TGG AGG ACT GTC CCT TTT G R[a]: TTG CAA CAA CCC ACT TGC TCC C	1.5		66
	3	F[a]: ATG GGA TGA CAG GAG GTG TTG R: ACC ATC CAT TGA GGA TGG AAG seq: AGG AGT TCC AGT TGT AGT TCG	1.5		66

T_m, Annealing temperature (Centigrade); seq, sequencing primer only; DMSO, dimethyl sulfoxide.
[a] PCR primers also used for sequencing.

and 5 and the intervening intron from the genomic DNA of the proband established that he was a compound heterozygote for these two changes. The cysteine at position 223 is highly conserved in mouse, rat, zebrafish, and pufferfish, whereas R297 is conserved in mouse and rat but not in zebrafish or pufferfish. Neither variant was detected in 180 Caucasian control chromosomes. The proband's mother and father were of Turkish-Cypriot and Jamaican descent, respectively. Only his mother and younger brother were available for further study, and both were found to be heterozygous for the R297L variant but homozygous wild-type with respect to C223. The C223R variant was thus most likely inherited from his father, although *de novo* mutation cannot be ruled out. Screening of 100 control Afro-Caribbean alleles failed to reveal any further C223R changes, whereas R297L was not detected on screening 90 control Turkish alleles.

Case history

The proband (46, XY, birth weight 2.98 kg) was born at term after an uneventful pregnancy. He was noted to have a micropenis and undescended testes at birth, and serum gonadotropins were undetectable at 2 months of age (LH < 0.5 mIU/ml, FSH < 0.5 mIU/ml). His penis grew reasonably in response to three injections of depot testosterone, but whereas his right testis was identified in the scrotum, his left testis remained undescended.

TABLE 3. Prevalence of common sequence variants in *GPR54* and *KISS1*

dbSNP ID	SNP	AA change	Cohort frequency (%)	Control frequency (%)
A. *GPR54*				
rs8111938	615A>C	None	1.5	0
rs350132	1091T>A	H364L	23	28
rs3746147	1155G>A	None	0	0
B. *KISS1*				
rs12998	207G>A	E20K	4.7	4.2
	256A>G	Q36R	9.4	4.2
rs1132112	287C>G	None	0	0
rs4889	391C>G	P81R	36	13.9
	565_566insA	Omits 7AA	76	85

AA, Amino acid; Freq., frequency.

At 15 months of age, he was admitted for further investigation of anterior pituitary function. His height/length was progressing consistently along the 50th percentile and weight along the 25th percentile. He had a normal GH response to glucagon stimulation [2.1 (basal) to 30.5 ng/ml (peak) (6.2–91.5 mU/liter; North-East Thames Radio-Immuno Assay, cut-off for the diagnosis of GH deficiency, 11.7 ng/ml]. He had a normal cortisol response to glucagon stimulation [19.8–47.0 μg/dl (554–1317 nmol/liter)], normal free T_4 [1.37 μg/dl (17.5 nmol/liter)] and normal TSH response to TRH stimulation (0.5–7.3 mU/liter). He had normal basal prolactin, and undetectable basal gonadotrophins (LH < 0.5 mIU/ml, FSH < 0.5 mIU/ml). Results of a 3-d human chorionic gonadotropin (hCG) stimulation test (1000 IU daily) showed a limited testosterone response to stimulation (Table 4A). An ultrasound scan at this time identified both testes and bilateral hydroceles. The right testis was soft, measured 9 × 6 × 10 mm, and was within the scrotal sac. The left testis had a maximum diameter of 7 mm and was identified at the left inguinal ring. A prolonged course of hCG stimulation produced some increase in penile size but failed to stimulate descent of the left testis. Therefore, herniotomy and orchidopexy of a very small left testis were performed.

His progress during childhood was uneventful. However, he showed progressive weight gain and linear growth from the age of 3.5 yr so that by 9.7 yr of age, he was relatively tall (height 148.6 cm, > 97th percentile; parental target height 25th percentile) and heavy (weight 49.9 kg, ≫ 97th percentile) but with a small penis and testes. Repeat endocrine evaluation at 10 yr of age showed a poor gonadotrophin response to LHRH stimulation (Table 4B) and suboptimal testosterone response to 3 wk stimulation with hCG (1000 IU twice weekly) (Table 4C). He was therefore started electively on low-dose testosterone replacement to induce puberty. No neuroimaging was performed.

The proband's mother, who was heterozygous for R297L, had experienced menarche at 11 yr of age. She has regular

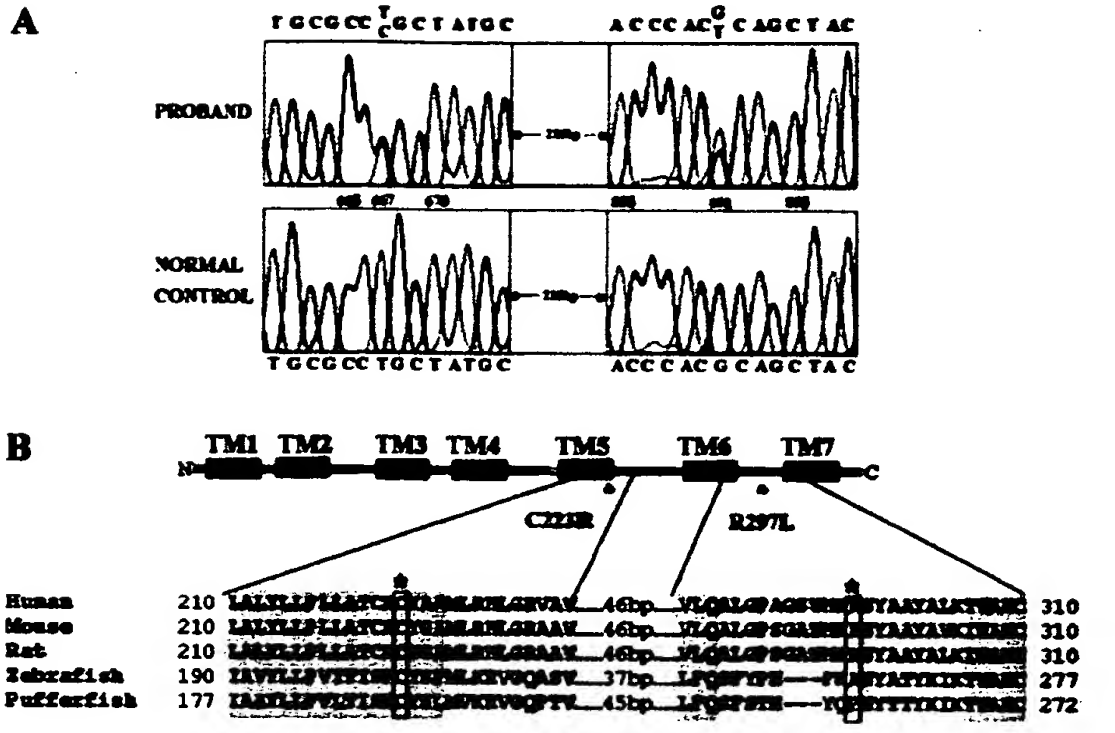

FIG. 1. Rare *GPR54* sequence variants found in propositus. TM, Transmembrane α helix.

menstrual cycles and detectable gonadotropins (LH 7.2 mIU/ml, FSH 8.3 mIU/ml). His father is believed to have normal reproductive function but was not available for testing. His younger brother, heterozygous for R297L but homozygous wild-type for C223 had normal genitalia and descended testes at birth. Of note, there was no known family history of hypogonadism or infertility in the father's eight siblings.

Characterization of mutant GPR54 function

Using the proprietary Flp-In system, isogenic cell lines stably expressing either wild-type GPR54 or one of the two mutant GPR54s at the same level of transcription were generated. A sensitive fluorometric calcium mobilization assay was then used as described to assess the response of each GPR54 species to the kisspeptin 112–121 decapeptide, previously established to be a potent agonist for GPR54 (12, 14). In our hands (Fig. 2), this assay is highly sensitive for detecting GPR54 activity: calcium flux above baseline for wild-type GPR54 is detectable at subnanomolar concentrations of ligand, with maximal flux achieved at around 100 nM.

The EC_{50} of the wild-type receptor was determined from 20 independent measurements. The (arithmetic) mean EC_{50} is 3.20 nM (lower 95% confidence interval 1.99 nM, upper 95% confidence interval 4.41 nM, SEM 0.56 nM). The range of EC50s was 0.2–9.8 nM. The C223R mutant was profoundly impaired in its signaling capability with an EC_{50} of 200 nM and maximal activity around 20% of wild-type GPR54. The function of R297L was only mildly affected, with a measured EC_{50} of 7 nM and a maximal activity of 85% of wild type (Fig. 2). A one-sample *t* test suggests this EC_{50} difference is significant, with $P < 0.0001$ and $t = 6.6$. Kolmogorov-Smirnoff deter-

TABLE 4. Results of dynamic endocrine testing

	A. hCG stimulation test at 15 months	
	Pre-hCG	Day 3 post-hCG
DHEA-S [μg/liter (μmol/liter)]	<385 (<1.0)	<385 (<1.0)
Androstenedione [ng/ml (nmol/liter)]	0.17 (0.6)	0.17 (0.6)
Testosterone [ng/ml (nmol/liter)]	<0.15 (<0.5)	0.45 (1.5)

	B. LHRH stimulation test at 10 yr		
	0 min	20 min	60 min
LH (IU/liter)	<0.7	1.0	<0.7
FSH (IU/liter)	<0.2	1.1	1.8

	C. hCG stimulation test at 10 yr		
	Pre-hCG	Day 3 post-hCG	Day 21 post-hCG
DHEA-S [μg/liter (μmol/liter)]	1001 (2.6)	905 (2.35)	1220 (3.17)
Androstenedione [ng/ml (nmol/liter)]	1.0 (3.6)	1.2 (4.1)	1.7 (5.8)
Testosterone [ng/ml (nmol/liter)]	0.21 (0.7)	0.3 (1.0)	1.4 (4.6)
Dihydrotestosterone [ng/ml (nmol/liter)]	<0.04 (<0.12)	<0.04 (<0.12)	N/A

All injections were supplied by the hospital and administered under supervision. DHEA-S, Dehydroepiandrosterone sulfate; N/A, not available.

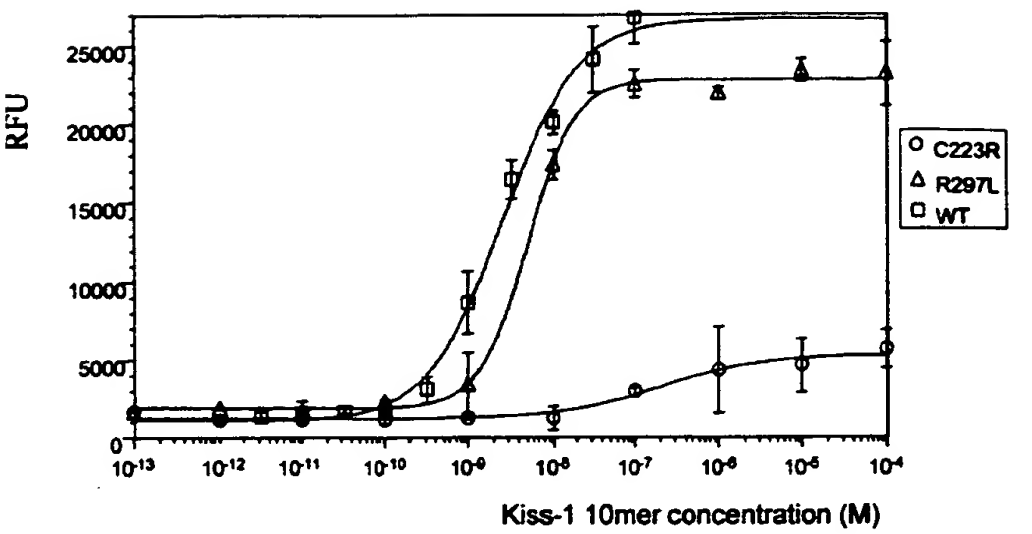

FIG. 2. Response of GPR54 variants to stimulation with kisspeptin 112–121 decapeptide. RFU, Relative fluorescence units. Experiments were performed in duplicate on 2 consecutive days, and SDs are indicated. Dose-response curves were generated using a four-parameter logistic equation. The resulting parameters and correlation coefficients are indicated.

$y = ((A - D)/(1 + (x/C)^B)) + D$:

	A	B	C	D	R^2
○ C223R	1169.893	0.711	2.04e-7	5304.917	0.969
△ R297L	1845.014	1.53	5.02e-9	22850.36	0.999
□ WT	997.683	0.965	2.54e-9	26693	0.994

mination of skewedness and kurtosis shows the control data fit a normal distribution, so a parametric *t* test is appropriate.

Discussion

Human GPR54 is a G protein-coupled receptor with homology to the family of galanin receptors, although galanin and galanin-like peptides do not bind the receptor with appreciable affinity. In the past year, homozygosity mapping and candidate gene analysis of large consanguineous pedigrees has led two groups to describe loss-of-function mutations in GPR54 in association with autosomal recessive HH (4, 5). In parallel, the independent generation and characterization of a *Gpr54* knockout mouse model, which shows a phenocopy of human isolated HH, has demonstrated that the function of GPR54 is conserved in mammals and that GPR54 is absolutely required for the normal function of this axis (5).

Mouse and human studies to date suggest GPR54 has a role in the release of GnRH (5, 6), further weight having recently been added to this hypothesis by the demonstration that peripherally and centrally administered kisspeptins can provoke robust LH and FSH release (6–8). However, it is not yet clear whether kisspeptins subserve primarily autocrine, paracrine, or endocrine function. Intriguingly, the putative endogenous GPR54 ligand, an amidated proteolytic product of full-length kisspeptin-1, sometimes called metastin (12–14), is normally present only at low picomolar concentrations in plasma but rises some 10,000-fold by the end of pregnancy (15). Both kisspeptin-1 and GPR54 are widely expressed, with the highest GPR54 expression being reported in placenta, brain, pituitary, pancreas, and spinal cord, whereas the highest kisspeptin-1 expression is seen in placenta, with significant levels also in pancreas and testis (12–14). This widespread expression implies a more complex role for GPR54/kisspeptin-1 than appreciated at present, and studies of humans harboring loss-of-function mutations are likely to make a valuable contribution to further elucidation of its biology.

The current report adds to the spectrum of loss-of-function mutations in GPR54. Previous studies reported five different mutations in four families or individuals with HH. Homozygosity for a 155-bp deletion spanning the splice acceptor site of the intron 4-exon 5 junction and part of exon 5 was detected in a consanguineous pedigree with five affected members (4). This homozygous deletion cosegregated with HH in this family and was inferred to result in loss of receptor function due to truncation of the coding sequence at residue 267. A second rare sequence variant, this time a missense mutation, L102P, immediately after the second transmembrane helix, was also found in one family on screening three other kindreds with familial HH. However, no cosegregation or functional studies of this mutant were reported (4). Another report of loss of function of GPR54 in patients with HH described both a homozygous L148S point mutation in a conserved region of the second intracellular loop in a consanguineous Saudi kindred and compound heterozygosity for two mutations, R331X and X399R, in an unrelated Afro-Caribbean patient, which were proven to result in significant loss of GPR54 signaling *in vitro* (5). In total, the published reports to date describe the screening of 68 unrelated probands with normosmic HH (five familial; 63 idiopathic) and normal GnRH receptor gene sequence, of whom three had two proven hypofunctional or nonfunctional GPR54 alleles (two familial; one idiopathic). This is consistent with our finding of one such patient in a further 17 studied (three familial; 14 idiopathic: Table 1). Cumulative results now suggest that, in those in which GnRHR coding sequence mutations have been ruled out, around 2–3% (two of 77 studied) of idiopathic normosmic HH may be accounted for by GPR54 loss-of-function mutations, whereas at present 25% (two of eight studied) of familial cases have been found to have such

mutations. However, no GPR54 mutations were identified in patients or families with CDP alone.

It is highly likely, although not conclusively proven, that compound heterozygosity for the C223R and R297L variants is the cause of the idiopathic HH seen in our proband. C233R is severely defective in its signaling capability and is not seen as a common variant in the Afro-Caribbean population. In contrast, the effect of the R297L mutation on signaling is only modest, although highly statistically significant, when studied *in vitro*. However it is a highly conserved residue in mammalian GPR54 and was not found in an ethnically matched control population. It is likely that its effects in combination with the severe dysfunction of the C233R variant are sufficient to lead to a clinical phenotype.

Although the patient we describe is not yet beyond the age of normal puberty, he had sufficient clinical and biochemical evidence of HH to warrant induction of puberty at an age appropriate for his peer group. For example, bilateral cryptorchidism and micropenis were noted at birth; he had undetectable gonadotropins at 2 months of age [when the hypothalamic-pituitary-gonadal axis is usually relatively active (16)]; and his response to exogenous GnRH stimulation at the age of 10 yr was poor. In addition, his testosterone response to stimulation for 3 wk with hCG at this time was relatively low. Although this may reflect the tropic effect of prolonged gonadotropin insufficiency or delayed orchidopexy, it remains possible that the GPR54/kisspeptin-1 system could have a direct effect on testicular function, too. Of note, GPR54 mRNA has previously been detected, albeit at low levels, in human testis (13, 14). Thus, the effect of recombinant gonadotropin stimulation on the induction of fertility in patients with *GPR54* gene mutations remains to be seen.

The present report uses a GPR54 signaling assay that is substantially more sensitive than that used to evaluate previous pathogenic mutations. The assay reported originally was based on transient expression of GPR54, stimulation with kisspeptin-1 112–121 decapeptide, and determination of the generation of inositol triphosphate. Stimulation of inositol triphosphate levels was seen only at around 10 nM kisspeptin-1 112–121 for wild-type GPR54, with maximal activity around 100 μM (3). In contrast, this study employed real-time measurements of calcium flux, similar in principle to those previously used in the identification of kisspeptin as a *bona fide* GPR54 ligand (12–14). As seen in Fig. 2, this assay detects signaling at subnanomolar concentrations of the kisspeptin decapeptide, and activity is maximal at around 100 nM, some 2–3 orders of magnitude lower than the ligand concentration required to maximize inositol triphosphate generation in the previous assay.

In the absence of functional assessment of the GPR54 L102P variant, GPR54 C223R and R297L are the second- and third-point mutations after L148S reported to result in impaired GPR54 function because the 155-bp deletion, R331X, and X399R are all likely to have resulted in loss of function largely through impaired expression of mRNA and/or protein product. The new mutations may thus prove more informative with respect to understanding of the molecular determinants of GPR54 signaling. The dramatic loss of function of GPR54 C223R is unsurprising in view of likely disruption of the fifth transmembrane helix. Indeed, it will be of interest to establish whether receptor protein is expressed at wild-type level at the plasma membrane and, if so, whether ligand binding is preserved. R297L is situated in the final extracellular loop of GPR54 in a slightly less conserved region, and its effect on signaling is consequently relatively mild *in vitro*. It remains to be determined whether its additional loss of function *in vivo* is due to inefficient receptor synthesis or processing. Nevertheless, the dose-response characteristics of this mutation raise the possibility that supraphysiological doses of exogenous kisspeptin or an analog could be used to restore GnRH pulsatility and possibly fertility in this patient. A similar approach, using high doses of GnRH, has been used to induce ovulation in a woman with a partial loss-of-function mutation in the GnRH receptor (17). Furthermore, because we have not been able to establish whether the father of the propositus is indeed heterozygous for the C223R variant, we cannot conclude on phenotypic grounds that heterozygosity for this variant is not clinically expressed. Thus, dominant negativity of GPR54 C223R, or an interaction between C223R and R297L, cannot be formally excluded. However, although there are precedents for dominant-negative GPR mutations, often based on receptor misrouting or G protein sequestration, the dominant negativity is often more apparent *in vitro* than *in vivo* (18), and we believe it more likely in this case that it is the cumulative loss of function of the two variants that is responsible for the clinical phenotype.

In addition to identifying and characterizing two rare sequence variants in the *GPR54* gene, we have also confirmed two previously described SNPs (Table 3A). One of these is nonsynonymous, with an alternative histidine to leucine change encoded in the cytoplasmic tail. A third SNP recorded in the dbSNP database was not confirmed (1155G>A; rs3746147). Analysis of *KISS1* revealed four single nucleotide polymorphisms, three nonsynonymous, and one not previously recorded. In addition, an insertional polymorphism that leads to truncation of the kisspeptin-1 protein product by seven amino acids was also confirmed (Table 3B). Once again, a further SNP recorded in the dbSNP database was not confirmed (287C>G; rs1132112). All three nonsynonymous KISS1 sequence variants lead to amino acid changes in the amino-terminal part of the molecule. This is not directly involved in signaling but is likely to contribute to the regulation of processing and secretion of the active peptide. We have documented the frequency of occurrence of these variants in our study population and also in 180 Caucasian control chromosomes. This information could be used in future to guide case control studies looking at either indices of reproductive function or fertility or possibly indices of tumor invasion or metastasis in patients with malignancy, in view of the considerable interest in KISS1 as a metastasis-suppressing gene.

In summary, we have described two novel missense mutations in the G protein-coupled receptor GPR54 that result in impaired kisspeptin-1 stimulation of calcium flux. Compound heterozygosity for these mutations is associated with HH, detected at birth in the proband due to bilateral cryptorchidism and micropenis. Although not common, loss-of-

function mutations in GPR54 appear to be a significant cause of HH in patients from diverse ethnic backgrounds.

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